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Development of Steroid Sulfatase Inhibitors

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Abbreviations: Adiol, androstenediol; AIs, aromatase inhibitors; DASIs, dual aromatase sulfatase inhibitors; DHEA-S, dehydroepiandrosterone sulfate; ER, estrogen receptor; E1S, estrone sulfate; hCAII, human carbonic anhydrase II; HDBC, hormone dependent breast cancer; SAR, structure-activity relationship; SERM, selective estrogen receptor modulator; STS, steroid sulfatase.

Abstract

Hydrolysis of biologically inactive steroid sulfates to unconjugated steroids by steroid sulfatase (STS) is strongly implicated in rendering estrogenic stimulation to hormone-dependent cancers such as those of the breast. Considerable progress has been made in the past two decades with regard to the discovery, design and development of STS inhibitors. We outline historical aspects of their development, culminating in the discovery of the first clinical trial candidate STX64 (BN83495) and other sulfamate-based inhibitors. The development of reversible STS inhibitors and the design of dual inhibitors of both aromatase and STS is also discussed.

Introduction

Since the 1970s, postmenopausal women with hormone-dependent breast cancer (HDBC) receiving adjuvant endocrine therapy have been given tamoxifen. This agent works as an anti-estrogen, or more precisely a SERM (selective estrogen receptor modulator), by principally antagonising the binding of estrogens to the estrogen receptor (ER) in the breast. Over three decades ago, another class of anti-endocrine agent was developed whose mechanism of action is to diminish the availability of estrogens to tumors through inhibiting the conversion of androstenedione to estrone by the aromatase enzyme in the final step of estrogen biosynthesis. Extensive research led to the successful development of three third-generation aromatase inhibitors (AIs) the non-steroidal letrozole and anastrozole, which inhibit aromatase selectively and reversibly, and the steroidal exemestane which is an irreversible inhibitor. All three AIs are well tolerated and widely used in the clinic for treating advanced breast cancer. Recently, several randomized comparative clinical trials have demonstrated the advantages of using anastrozole, letrozole, and exemestane over tamoxifen as first-line agents in the treatment of patients with primary tumors (Mouridsen et al., 2001; Bonnetterre et al., 2001; Paridaens et al., 2003; Mouridsen et al., 2003; Milla-Santos et al., 2003; Nabholz et al., 2003a; Nabholz et al., 2003b). These findings, therefore, widen not only the indications for these three AIs, but also the population of patients who may benefit from receiving these agents.

While it has been demonstrated that the use of AIs is an effective strategy for reducing estrogenic stimulation to hormone responsive tumors, the overall response rates and clinical benefit rates were found to vary from 21 to 33% and 49 to 59% respectively in randomized first-line studies of third generation AIs in metastatic disease (Smith and Dowsett, 2003). These clinical findings have suggested that a substantial population of ER-positive tumors are not responding to aromatase inhibition.

It became evident more than two decades ago that the steroid sulfatase (STS) pathway could also be an important source of estrogens. Evidence to support this hypothesis includes (i) a million-fold higher STS activity than aromatase activity in liver and normal and malignant breast tissues (James

et al., 1987) , (ii) the origin of E1 from E1S in breast cancer tissue is about 10 times more than that from androstenedione (Santner et al., 1984), and iii) STS expression is an important prognostic factor in human breast carcinoma (Utsumi et al., 1999; Suzuki et al., 2003). Most estrogens that originate from the aromatase pathway are converted to and stored in the body as sulfates. However, this reservoir of estrogen sulfates, though biologically inactive *per se*, could significantly contribute to the overall estrogenic stimulation of tumors when STS catalyzes the hydrolysis of substrates such as estrone sulfate (E1S) to estrone (E1), and dehydroepiandrosterone sulfate (DHEA-S) to DHEA. The formation of DHEA by such a route constitutes the production of 90% of androstenediol (Adiol) which is an estrogen (ca. 100-fold weaker than estradiol), despite structurally being classified as an androgen (Adams et al., 1981; Dauvois and Labrie, 1989; Naitoh et al., 1989; Chetrite et al., 2000). (For further discussion refer to Purohit et al. ‘Sulfatase as a pivotal player in estrogen synthesis and metabolism’ in this issue). Thus, STS is an attractive and novel target for therapeutic intervention of hormone-dependent cancers such as that of the breast.

Considerable progress has been made in the past two decades with regard to the development of STS inhibitors. This article outlines historical aspects of this development, culminating in discovery of the first clinical trial candidate STX64 (BN83495) and other sulfamate-based inhibitors. Development of reversible STS inhibitors and the emergence of dual inhibitors of both aromatase and STS will also be discussed.

Estrone sulfate analogues and steroidal sulfamate-based inhibitors

With E1S being the natural substrate of STS, the most obvious target was to modify this steroid conjugate in the initial search for inhibitors, ideally by designing non-hydrolysable sulfate group surrogates. Early work, however, focused upon keeping the sulfate group intact, but replacing the estrone backbone with other templates that may interact with the active site of STS. This gave rise to several series of weak to modest inhibitors represented by 5-androstene-3 β ,17 β -diol-3-sulfate (Evans et al., 1991), flavonoid daidzein 4'-O-sulfate and daidzein 4',7-di-O-sulfate (Wong and Keung,

1997), and 2-(hydroxyphenyl)indole sulfates (Birnböck and von Angerer, 1990). However, these agents would be of little value clinically because they are, in principle, alternative substrates for STS as their sulfate group(s) are expected to be hydrolysed by the enzyme. This potential metabolism of the inhibitor by STS will pose a particular problem especially when the compound released has undesirable endocrinological activities such as estrogenicity.

Keeping to the modification of E1S, the alternative strategy we adopted is to retain the estrone backbone but to replace the sulfate group (OSO_3^-) of E1S with other sulfate surrogates or mimics such as phosphate (Anderson et al., 1995), phosphonates [$-\text{OP}(=\text{O})(\text{OH})\text{Me}$] and thiophosphonates [$-\text{OP}(=\text{S})(\text{OH})\text{Me}$] (Purohit et al., 1994; Duncan et al., 1993; Howarth et al., 1993; Howarth et al., 2002), sulfonates ($-\text{OSO}_2\text{R}$) (Howarth et al., 1997, Li et al., 1995), sodium methylenesulfonate ($-\text{CH}_2\text{SO}_3^-\text{Na}^+$) (Li et al., 1995), sulfonyl halides ($-\text{SO}_2\text{Cl}$ and $-\text{SO}_2\text{F}$) (Li et al., 1993), sulfonamide ($-\text{SO}_2\text{NH}_2$) (Li et al., 1993; Dibbelt et al., 1994), and the methylsulfonyl group ($-\text{SO}_2\text{CH}_3$) (Li et al., 1993; Dibbelt et al., 1994) for example. Most of these E1 derivatives were designed not to be metabolically labile or hydrolysable by the enzyme like sulfated compounds in the course of competing with E1S for binding to the enzyme active site. Despite the large number of compounds that had been explored in this category, none of these compounds was deemed to be potent enough or adequately attractive pharmaceutically for further development as an inhibitor of STS.

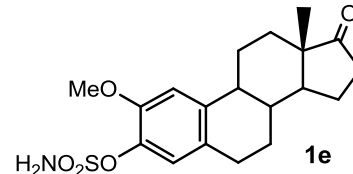
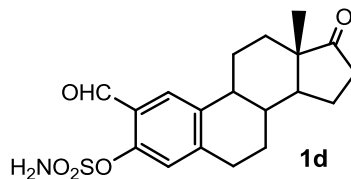
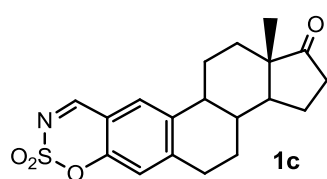
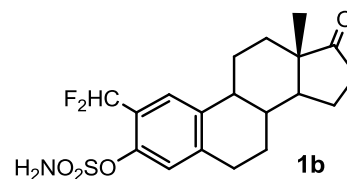
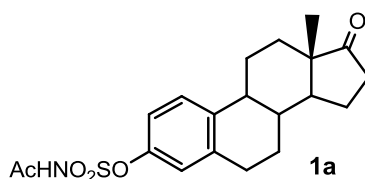
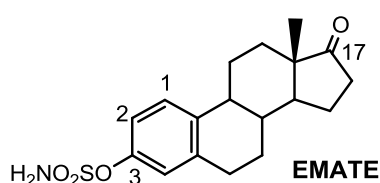


Fig. 1 Examples of steroidal sulfamate-based STS inhibitors: EMATE and A-ring modified derivatives **1a** – **1e**.

The breakthrough in the design of potent STS inhibitors came when the sulfate group of E1S was replaced by a sulfamate moiety ($-\text{OSO}_2\text{NH}_2$). Estrone 3-*O*-sulfamate or EMATE (Fig. 1) was found to be a highly potent inhibitor with an IC_{50} of 18 nM in a human placental microsomes preparation. But more exceptionally, EMATE was found to inhibit STS in a time- and concentration-dependent manner, indicating that the mechanism of action is of an irreversible nature. Subsequent work has confirmed that the pharmacophore for potent and irreversible inhibition of STS is a phenol sulfamate ester or an aryl sulfamate, ie. $\text{Ar-OSO}_2\text{NH}_2$. It is important that the *N*-atom of the sulfamate group is unsubstituted, as several alkylated (Howarth et al., 1994; Woo et al., 1997) and acylated (Woo et al., 1997) derivatives of EMATE were found to be weak inhibitors of STS. In one study, only *N*-acetylEMATE (**1a**, Fig. 1) showed irreversible STS inhibition, albeit less potently and efficiently than EMATE (Woo et al., 1997). The hydrolyzable S-O bond of the sulfamate group is also important for potent inhibition of STS since the *S*-sulfamoyl ($-\text{SSO}_2\text{NH}_2$) and sulfamido ($-\text{NHSO}_2\text{NH}_2$) derivatives of EMATE were significantly weaker inhibitors (Woo et al., 1996a). It is thought that during irreversible inhibition the sulfamoyl group is transferred to a residue in the STS active site.

EMATE was found to be orally active *in vivo*. In one study (Purohit et al., 1995), administration of EMATE at 10mg/kg inhibited rat liver STS activity almost completely (99%) when given by the oral or subcutaneous route and the inhibition persisted (>95%) for up to 7 days after a single dosing of EMATE. However, EMATE was unexpectedly shown to be more estrogenic than ethinylestradiol when administered orally in rats (Elger et al., 1995), although it was revealed subsequently that estrogen sulfamates per se did not bind to the estrogen receptor (Elger et al., 2001). A follow-up study demonstrated that STS has a crucial role in regulating the estrogenicity associated with EMATE as its estrogenicity is abolished when STS is inhibited by the co-administration of a non-

estrogenic STS inhibitor (Chander et al., 2004). Nonetheless, this undesirable property of EMATE rendered the inhibitor unattractive for use as an anti-endocrine agent for HDHC, although its congener, estradiol 3-*O*-sulfamate, entered human clinical trials in the late 90s as an orally active pro-drug for estradiol.

The issue of estrogenicity with EMATE was a setback for an otherwise highly potent STS inhibitor. Much subsequent efforts were principally focused on designing EMATE analogues that are either devoid of, or have a significantly reduction in, estrogenicity but at the same time retain high potency against STS. The initial strategy adopted involved the introduction of substituents such as allyl, *n*-propyl, nitro, cyano, methoxy and halogens to the 2- and/or 4-position of the A-ring of EMATE. From this work, compounds possessing bulkier aliphatic groups were found to be weaker STS inhibitors, whereas electron-withdrawing substituents, namely halogens, showed higher potency than EMATE against STS in vitro (Purohit et al., 1998). One compound, 2-difluoromethylestrone 3-*O*-sulfamate (**1b**, Fig. 1), was found to have an IC₅₀ of 100 pM against STS in a placental microsomes preparation, which was some 90-fold lower than that of EMATE obtained from the same assay (Reed et al., 2004).

A series of steroidal oxathiazine STS inhibitors was developed of which estra-1,3,5(10)-trien-17-one-[3,2,*e*]-1',2',3'-oxathiazine-2',2'-dioxide (**1c**, Fig. 1) was found to inhibit the STS activity in a MCF-7 cells preparation with an IC₅₀ of 9 nM (Peters et al., 2003). However, it is not entirely clear whether the inhibitory activity observed is due to **1c** itself or its synthetic precursor 2-formyl-EMATE (**1d**, Fig. 1), which is a tentative product upon the hydrolysis of **1c** in situ. When **1c** was administered intra-peritoneally daily to nude mice bearing xenografts MCF-7 tumors, a significant reduction in the tumor size over a 5-week period was reported, although the inhibitor concentration used was not disclosed. With regard to estrogenicity, **1c** was reported to show greatly reduced activity.

The most successful and significant A-ring modified analogue of EMATE is considered to be 2-methoxyestrone 3-*O*-sulfamate (2-MeOEMATE, **1e**, Fig. 1). 2-Methoxyestradiol (Panzem[®]), the

congener of 2-methoxyestrone, which is the parent steroid of **1e**, is an anti-proliferative/anti-angiogenic microtubule disruptor that has reached clinical trial (Tevaarwerk et al., 2009). While the STS inhibitory activity of **1e** in a placental microsomes preparation was found to be similar to EMATE ($IC_{50} = 30$ nM), it is in contrast non-estrogenic (Purohit et al., 1999). More interestingly, in addition to its STS inhibitory activity, **1e** was found to induce apoptosis in several cancer cell lines and to inhibit tumor angiogenesis (Leese et al., 2005; MacCarthy-Morrogh et al., 2000, Newman et al., 2004, Chander et al., 2007), as well as possessing excellent bioavailability. Hence, **1e** has potential applications for the treatment of both HDBC and also other forms of hormone-independent cancers.

Because of the relative inaccessibility to synthetic manipulations, the B- or C-ring of EMATE has generally not been specifically targeted for modification. In contrast, the D-ring of EMATE has been widely modified, not only for attenuating the estrogenicity of the parent compound but also for generating many different structural classes of inhibitors.

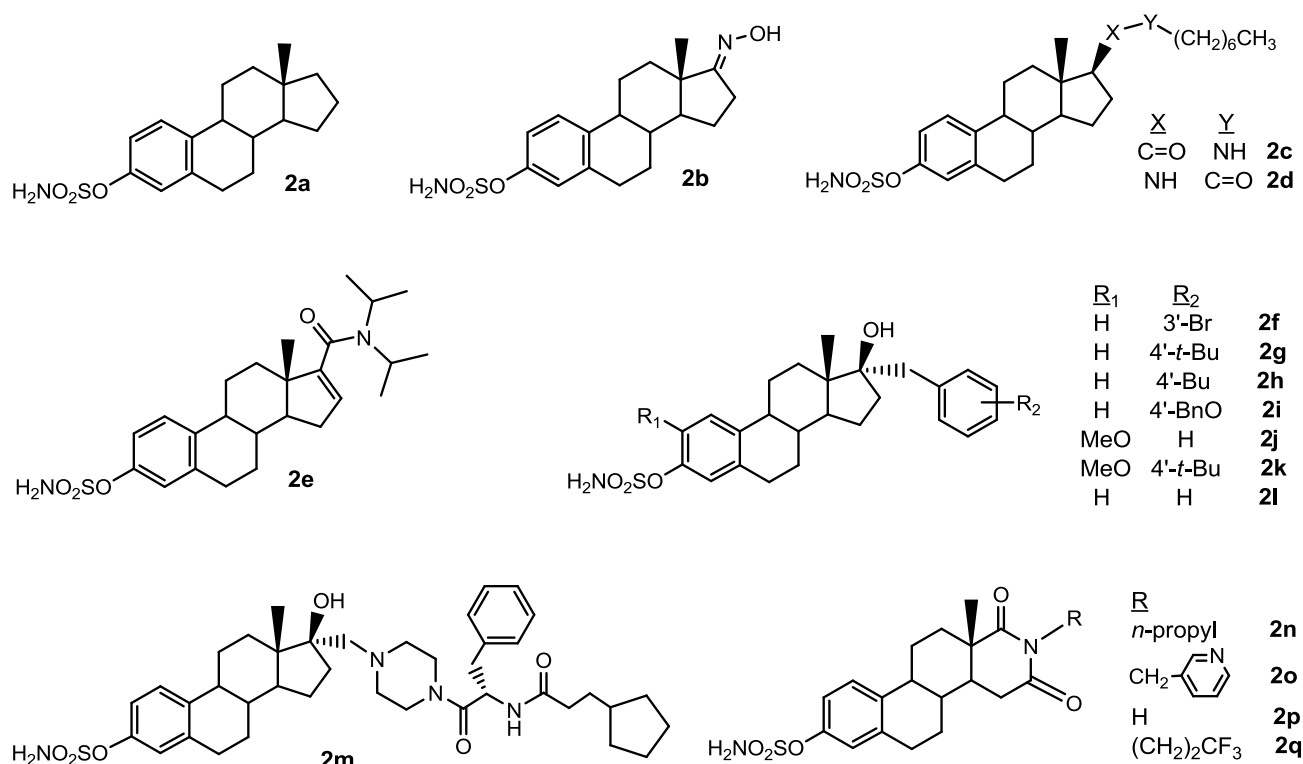


Fig. 2 Examples of D-ring modified steroidal and steroid-like sulfamate-based STS inhibitors.

When the carbonyl group of EMATE was removed upon reduction to a methylene group, the resulting derivative (**2a**, Fig. 2) was less estrogenic, but as potent as EMATE as an STS inhibitor (Purohit et al., 1999). Unexpectedly, when the carbonyl group of EMATE was replaced by an oxime, the resulting (*E*)-17-oximino derivative (**2b**, Fig. 2) inhibited STS to the same extent as EMATE *in vitro* (>99% inhibition at 0.1 μ M in MCF-7 breast cancer cells) and *in vivo* but showed enhanced estrogenicity as **2b** stimulated uterine growth in ovariectomized rats some 1.5 times stronger than EMATE (Hejaz et al., 1999).

When hydrophobic substituents were introduced via a linker to the 17 β -position of EMATE, replacing its carbonyl group on the D-ring entirely, an increase in potency and a reduction in estrogenicity of the resulting derivatives was observed. Li et al. reported 17 β -(*N*-alkylcarbamoyl)- and 17 β -(*N*-alkanoyl)estradiol 3-*O*-sulfamates to be highly potent STS inhibitors with the *n*-heptyl derivatives from both series (**2c** and **2d**, Fig. 2) showing optimal inhibitory activities (IC₅₀ value in MDA-MB-231 cells = 0.4 nM) (Li et al., 1998). When the estrogenicity of these compounds was assessed using the estrogen-sensitive MCF-7 cell line that proliferates upon stimulation by estrogens, neither inhibitor exhibited estrogenic activity at a concentration of 1 μ M, which was about 2000 times higher than their IC₅₀ values against STS. It is therefore evident that the introduction of hydrophobic side-chains to EMATE has a dual effect of increasing the binding of the resulting compounds to the enzyme active site and abolishing estrogenicity of the parent phenolic compound.

Li et al. further expanded and optimized the 17 β -(*N*-alkylcarbamoyl) derivatives of EMATE to give a series of α,β -unsaturated amides of which 17-(*N,N*-diisopropylcarbamoyl)estra-1,3,5(10),16-tetraene-3-*O*-sulfamate (KW-2581, **2e**, Fig. 2) is the most widely reported and developed (Ishida et al., 2007a, 2007b, 2008). Structurally, this compound differs from a 17 β -(*N*-alkylcarbamoyl) derivative of EMATE by having a double bond between C16 and C17 of the D-ring. According to the synthetic route developed by Li et al. (Li et al., 1998), an 17-(*N*-alkylcarbamoyl)estra-

1,3,5(10),16-tetraene-3-*O*-sulfamate is a precursor which upon hydrogenation of the double bond yields an 17 β -(*N*-alkylcarbamoyl)estrone 3-*O*-sulfamate. When tested in the hormone receptor-positive human breast cancer cell line ZR-75-1, **2e** inhibited STS activity with an IC₅₀ of 13 nM (Ishida et al., 2007a). In another assay, **2e** inhibited recombinant human STS activity with IC₅₀s of 2.9 nM and 4.2 when E1S and DHEA-S were used as substrate respectively. Like EMATE, the inhibition was found to be time- and concentration-dependent (Ishida et al., 2008). Upon oral administration, **2e** effectively induced regression of E1S-stimulated tumor growth in a nitrosomethylurea-induced rat mammary tumor model (Ishida et al., 2007b). The failure to stimulate the growth of MCF-7 cells as well as in uteri in ovariectomized rat indicated the lack of estrogenicity of **2e** (Ishida et al., 2007b).

Alongside EMATE, earlier work also showed that its congener estradiol 3-*O*-sulfamate (E2MATE) was as potent as an STS inhibitor in vitro (Reed et al., 1996). Employing a stereoselective addition of Grignard reagents to the C17-carbonyl group of E1 followed by sulfamylation, a series of 17 α -derivatives of estradiol 3-*O*-sulfamate were obtained of which 3'-bromobenzyl (**2f**, Figure 2), 4'-*t*-butylbenzyl (**2g**, Figure 2), 4'-butylbenzyl (**2h**, Figure 3) and 4'-benzyloxybenzyl (**2i**, Figure 2) derivatives were found to provide the most potent STS inhibition in a JEG-3 cells preparation (IC₅₀ values of 24, 28, 25 and 22 nM respectively) (Boivin et al., 2000). Unfortunately, 17 α -4'-*t*-butylbenzyl-E2MATE (**2g**) was subsequently shown to be estrogenic *in vivo*, rendering this inhibitor unsuitable for further therapeutic exploitation (Ciobanu et al., 2002, 2003a). In order to overcome this setback on estrogenicity, 2-methoxy-17 α -benzylestradiol-3-*O*-sulfamate (**2j**, Figure 2) and 2-methoxy-17 α -4'-*t*-butylbenzylestradiol-3-*O*-sulfamate (**2k**, Figure 2) were prepared (Ciobanu et al., 2003b), after recognising that substitution at the 2-position of EMATE with a methoxy group abolished estrogenicity but retained potency against STS (Raobaikady et al., 2003). As anticipated, both agents were found to show comparable potency to their corresponding 2-unsubstituted counterparts in the inhibition of STS activity in homogenates of transfected HEK-293

cells. In vivo, the 2-methoxy-17 α -benzyl-derivative (**2j**) showed no estrogenic activity in ovariectomized mice and efficiently blocked uterine growth induced by E1S.

Further expansion of 17 α -substituted analogues gave a series of *N*-derivatized 17 α -piperazinomethyl derivatives of estradiol 3-*O*-sulfamate (Ciobanu and Poirier, 2003). The best STS inhibitor was **2m** (Fig. 2). At a 1 nM test concentration, **2m** inhibited STS activity in homogenates of HEK-293 cells transfected with STS by 94%. This level of activity was close to that obtained with 17 α -benzyl-E2MATE (**2l**, Fig. 2) and 17 α -4'-*t*-butylbenzyl-E2MATE (**2g**, Fig. 2).

Poirier et al. continued to explore the substitution of the D-ring of EMATE and produced two libraries of compounds containing at the 16 β -position a propylene linker coupled with a different combination of hydrophobic amino acid and carboxylic acids. These compounds were also designed for inhibiting the steroidogenic enzyme type 1 17 β -hydroxysteroid dehydrogenase. No detailed biological results were reported, but it was claimed that 17 compounds possessed inhibitory activities comparable to or above the 78% inhibition of STS activity observed for EMATE in a homogenate of human embryonic kidney (HEK)-293 cells transfected with a STS expression vector (Poirier et al., 2002).

A D-ring enlargement of EMATE gave a series of novel *N*-substituted piperidinedione derivatives, which structurally are more accurately described as steroid-like. Two compounds, the *N*-(propyl) (**2n**, Fig. 2) and *N*-(1-pyridin-3-ylmethyl) (**2o**, Fig. 2) derivatives, showed exceptionally high potency, with both sharing the same IC₅₀ value of 1 nM in a human placental microsome preparation (cf. EMATE, 18 nM) (Fisher et al., 2003a). The *N*-unsubstituted derivative (**2p**, Fig. 2) showed similar potency (IC₅₀ = 20 nM) to EMATE, indicating that the six-membered piperidinedione ring is a good mimic of the D ring of EMATE. After an oral dose of 10 mg/Kg/d for 5 days, compounds **2n** and **2o** were found to inhibit rat liver STS by 99% (Fisher et al., 2003b). Both compounds were devoid of estrogenic activity in the rat uterine weight gain assay. In a subsequent *in vivo* study, **2n** was found to inhibit STS activity for a significantly longer duration than the non-steroidal STS inhibitor STX64 (Foster et al., 2006).

When the *N*-propyl group of **2n** was replaced with a *N*-3,3,3-trifluoropropyl group (Woo et al., 2008), the resulting compound **2q** (Fig. 2) gave an IC₅₀ of 180 pM against STS in JEG-3 cells which is 5-fold more potent than **2n** obtained from the same assay, completely inhibited rat liver STS activity after a single oral dose of 0.5 mg/kg, and, more interestingly, exhibited a significantly longer duration of inhibition over **2n** with a 50% recovery after 17 days (Foster et al., 2008). These distinctive biological properties of **2q** can be attributed to the increased lipophilicity and metabolic stability of the molecule rendered by its trifluoropropyl group, and also the potential H-bonding between its fluorine atom(s) and Arg98 in the active site of human STS, as suggested by molecular docking studies.

Sulfamate-based non-steroidal inhibitors

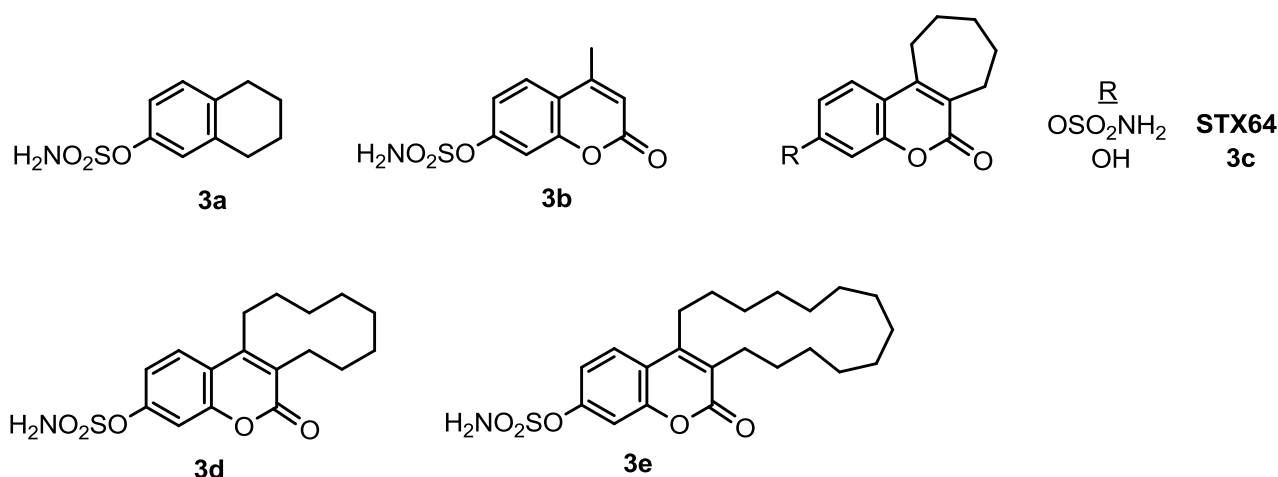


Fig. 3. Bicyclic and tricyclic non-steroidal STS inhibitors **3a** – **3e**.

When EMATE was found to be estrogenic, the most logical approach for addressing the shortcoming of a steroidal inhibitor was to design non-steroidal mimics of EMATE. It has long been recognised that non-steroidal agents themselves and their metabolites are less likely to exhibit unwanted endocrinological activities in vivo than their steroidal counterparts. When EMATE was found to have memory-enhancing effect in rats (Li et al., 1995; Li et al., 1996a) recognising that STS may regulate part of the immune response in human (Foulkes et al., 1997) and that STS may be

involved in the development of androgen-dependent skin disorders such as acne (Chen et al., 2002), there was an overwhelming case for developing non-steroidal STS inhibitors. Clearly, drugs that are steroidal will not be desirable for use in the treatment of some of these non-malignant conditions.

An initial attempt to design a non-steroidal inhibitor gave the A/B ring mimic of EMATE, tetrahydronaphthalene 2-*O*-sulfamate (**3a**, Fig. 3), which was found to exhibit STS inhibitory activity, but to a much weaker extent than EMATE (Howarth et al., 1994). In pursuit of alternative non-steroidal mimics of EMATE, a series of bicyclic coumarin sulfamates was synthesized, of which 4-methylcoumarin 7-*O*-sulfamate (**3b**, Fig. 3) showed an IC_{50} of 380 nM in an MCF-7 cells preparation, about 3-fold more potent than **3a**, but was still much weaker than EMATE (IC_{50} = 65 pM from the same assay) (Woo et al., 1996b). Further extension of the coumarin sulfamate series established that derivatives with hydrophobic substituents introduced at the 3- and/or 4-positions of the coumarin ring system were more potent STS inhibitors (Woo et al., 1998). On exploiting the flexibility provided by the Pechmann synthesis conditions for this scaffold, a series of tricyclic coumarin sulfamates was developed by reacting resorcinol with cyclic β -keto esters in the first step. When 2-oxo-1-cycloheptane carboxylate was employed, STX64 (BN83495, Fig. 3) was obtained, which was found to inhibit STS with an IC_{50} value of 8 nM (placental microsomes), some 3-fold more potent than EMATE (Woo et al., 2000; Purohit et al., 2001). This higher potency observed for STX64 compared with EMATE could be attributed to the higher affinity of STX64 for the enzyme site, since it was found that the apparent K_i value for STX64 (40 nM) is significantly lower than that for EMATE (670 nM) (Woo et al., 2000). In addition, it was postulated that the coumarin ring through conjugation renders the phenolic coumarin precursor (**3c**, Fig. 3) of STX64 a better leaving group than E1 [pK_a of phenol ca. 8.5 for **3c** vs ca. 10 for E1] and hence facilitates the “sulfamoylation potential” of STX64, which relates to the potency of the inhibitor in inactivating the enzyme. In vivo, a single dose (10mg/kg, p.o.) of STX64 inhibited rat liver STS activity by 93% (Purohit et al., 2000). When administered to rats bearing a nitrosomethylurea-induced mammary tumor, STX64 caused regression of E1S-stimulated tumor growth in a dose-dependent manner

(Purohit et al., 2000). Like EMATE, STX64 is an irreversible inhibitor of STS (Woo et al., 2000) and is active by the oral route but, in contrast, is devoid of estrogenicity (Purohit et al., 2000). STX64 had an oral bioavailability of 95% in rats and could be detected in plasma for up to 8 h (Ireson et al., 2004). The pharmacokinetics observed for STX64 can be attributed in part to the sequestration of the inhibitor into erythrocytes through binding to human carbonic anhydrase II (hCAII) (Ireson et al., 2004) and the X-ray crystal structure of STX64 in complex with hCAII was solved (Lloyd et al., 2005a).

STX64 is now in formal clinical development and has been evaluated in a “first-in-class” Phase I clinical trial for the treatment of patients with advanced breast cancer (Stanway et al., 2006, 2007). The clinical data from this trial showed that a treatment protocol consisting of either a 5 mg or a 20 mg dose of STX64 resulted in potent inhibition of STS activity in peripheral blood lymphocytes and biopsied breast tumour tissue without showing any serious drug-related adverse events. More significantly, stable disease was observed in 5 out of 8 evaluable patients. STX64 is now in Phase I/II trials in breast cancer and Phase II trials in endometrial and prostate cancers. In addition to oncological applications, STX64 has potential use in endometriosis (Purohit et al., 2008a, Fusi et al., 2008) and in skin disorders (Purohit et al., 2008b; Reed et al., 2008). The STS inhibitor PGL2001 is currently in Phase I trials in endometriosis. Recent data from ongoing clinical trials employing a new solid dosage formulation of STX64 [BN83495] in metastatic breast cancer patients have shown further evidence of stable disease and an impressive clinical decrease in androstenediol levels (Coombes et al., 2009).

Upon extension of the tricyclic coumarin sulfamate series, it was shown that *in vitro* inhibitory activity is the highest with **3d** (Fig. 3) (IC_{50} = 1 nM, placental microsomes), although this compound was found to be only marginally more potent than STX64 *in vivo* (Malini et al., 2000). Despite its relatively weak activity *in vitro* (IC_{50} = 370 nM, placental microsomes), **3e** (Fig. 3), however, was found to be the most potent tricyclic coumarin sulfamate *in vivo*, inhibiting rat liver E1-STS activity

by 23 and 94% when assayed 24 h after administration of the 0.1 and 1 mg/kg doses (Malini et al., 2000), presumably explained by depot effect relating to its high log P value.

Either in parallel or as a consequence of the discovery of STX64, a range of structurally diverse aryl sulfamate-based non-steroidal compounds with varying degree of STS inhibitory activity has been reported by several groups. The more interesting and notable compounds are shown in Fig. 4.

A series of (*p*-*O*-sulfamoyl)-*N*-alkanoyl tyramines was reported, of which the *N*-tetradecanoyl derivative **4a** was found to have an IC₅₀ value of 55.8 nM against STS in a placental microsomes preparation (Li et al., 1996b). Further work demonstrated that the inhibition by this compound is irreversible (Selcer et al., 1997). The aryl sulfamate moiety, the amido functionality and the length of the alkylene linker between the *p*-sulfamoyloxyphenyl ring and the amido group were found to be crucial to the SAR (structure-activity relationship) of these compounds (Chu et al., 1997; Kolli et al., 1999).

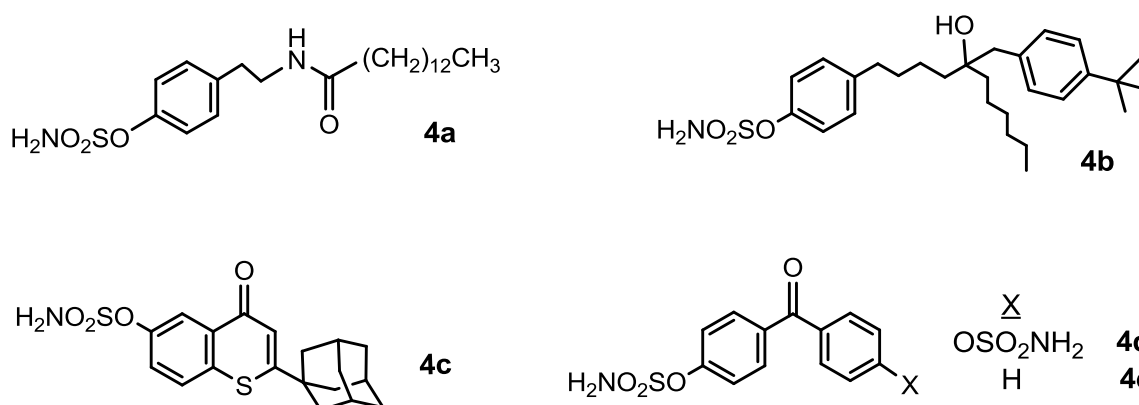


Fig. 4. Examples of non-steroidal STS inhibitors.

After the finding that 17 α -4'-*t*-butylbenzyl-E2MATE (**2g**, Fig. 2) inhibited STS with high potency, a series of 4-substituted monoaryl sulfamates was designed as non-steroidal mimics of **2g**. One compound **4b** (Fig. 4) showed optimal inhibitory activity with an IC₅₀ of 0.4 nM in homogenates of HEK-293 cells transfected with STS. EMATE inhibited STS with an IC₅₀ of 0.9 nM in the same assay (Ciobanu et al., 2002).

A series of chromenone- and thiochromenone-based sulfamates were reported, of which **4c** (Fig. 4) showed an IC_{50} value of 0.34 nM in purified STS, rendering this compound about 170-fold superior to EMATE (IC_{50} = 56 nM in the same assay) (Nussbaumer et al., 2002a). This exceptionally high potency observed for **4c** can be attributed to its aryl sulfamate moiety mimicking the A-ring of EMATE with the 1-adamantyl group concurrently exploiting hydrophobic interactions within the active site of STS. However, **4c** was estrogenic because it stimulated the growth of MCF-7 breast cancer cells by 99% at 100 nM (Nussbaumer et al., 2003).

Unexpectedly, benzophenone 4,4'-bissulfamate (**4d**, Fig. 4) showed an IC_{50} value of 190 nM (cf. 56 nM for EMATE) against recombinant human STS suggesting that this simple compound is a potent STS inhibitor (Nussbaumer et al., 2002b). SAR studies have shown that the bis-sulfamate is crucial to high potency, since the monosulfamate derivative, i.e. benzophenone 4-sulfamate (**4e**, Fig. 4) is about 25 times less active. These results were independently confirmed by another group in a separate report (Hejaz et al., 2004).

To the best of our knowledge, none of the aforementioned sulfamoylated non-steroidal inhibitors has undergone pre-clinical development. STX64 remains the most developed and studied sulfamate-based non-steroidal inhibitor to date.

Reversible inhibitors

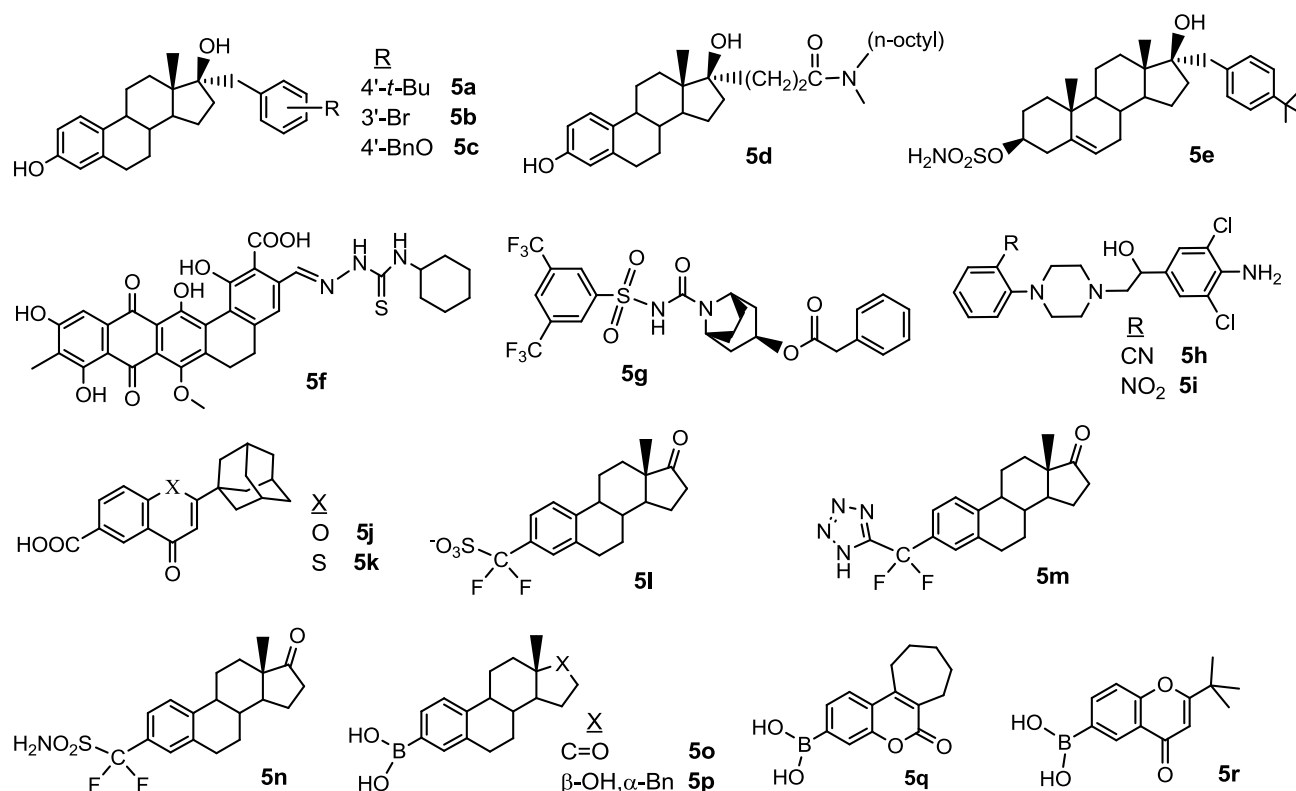


Fig. 5. Examples of reversible STS inhibitors.

Despite the great success achieved in the development of targeted irreversible STS inhibitors, several groups were inspired to design and discover reversible inhibitors of STS.

Although a significant number of reversible inhibitors were reported prior to or after the discovery of EMATE as part of the discovery programme or as a result of SAR studies, the first focused attempt in designing compounds for reversible inhibition was reported by Poirier *et al.* Derivatives of estradiol bearing a 17 α -4'-*t*-butylbenzyl (**5a**, Fig. 5), 17 α -3'-bromobenzyl (**5b**, Fig. 5), 17 α -4'-benzyloxybenzyl (**5c**, Fig. 5) or 17 α -*N*-octylpropanamide (**5d**, Fig. 5) substituent were among the most potent reversible inhibitors reported to date, with IC₅₀ values (JEG-3 cells) between 22 and 80 nM (Poirier *et al.*, 1998; Boivin *et al.*, 1999, 2000). Compound **5a** was about 7-fold weaker than EMATE against STS activity in a transfected HEK-293 preparation (Boivin *et al.*, 2000). Some of these phenolic compounds were sulfamoylated subsequently and the sulfamates were found to be significantly more potent as STS inhibitors (see previous section), confirming that the key pharmacophore for highly potent STS inhibition is an aryl sulfamate.

Several sulfamates of C19 (androstene) or C21 (pregnene) derivatives were prepared in an attempt to overcome the undesirable estrogenicity of some 17 α -substituted derivatives of EMATE. 17 α -*t*-Butylbenzyl-5-androsten-17 β -ol (**5e**, Fig. 5) is the best reversible inhibitor with an IC₅₀ of 46 nM in a homogenate preparation of HEK-293 cells and shows no estrogenic or androgenic properties in vitro (Ciobanu et al., 2003a).

Moving away from steroidal structures, a series of thiosemicarbazone derivatives of madurahydroxylactone was studied and reported to possess inhibitory activity against STS. The best agent, the cyclohexylthiosemicarbazone derivative (**5f**, Fig. 5) inhibited STS noncompetitively with a *K_i* value of 0.35 μ M and an IC₅₀ value of 460 nM in a placental microsome preparation (Jutten et al., 2002). In addition, a series of nortropinyl-arylsulfonylurea derivatives were prepared, of which **5g** (Fig. 5) inhibited STS in a purified enzyme assay with an IC₅₀ value of 0.084 μ M (*cf.* EMATE 0.056 μ M) (Nussbaumer et al., 2003b). Separately, researchers at Bayer identified from their compound library aryl piperazines **5h** and **5i** (Fig. 5), which inhibited STS in a STS protein preparation with IC₅₀ values of 48 and 78 nM, respectively (Lee et al., 2003). On reporting that chromenone- and thiochromenone-based sulfamates are potent inhibitors of STS, further SAR work was carried out in an attempt to replace the sulfamate group of these compounds with other surrogates. Two compounds 2-(1-adamantyl)-4-chromenone-6-carboxylic acid **5j** and 2-(1-adamantyl)-4-(thio)chromenone-6-carboxylic acid **5k** (Fig. 5) were found to be potent STS inhibitors. In a cell-free system using purified human STS, both inhibitors showed similar *K_i* values (0.50 μ M for **5j** and 0.53 μ M for **5k**) although **5k** was superior to **5j** (IC₅₀ = 0.18 μ M vs 9.4 μ M) in a cellular assay system using CHO cells overexpressing STS. Both **5j** and **5k** were chemically stable and showed no estrogenic potential (Horvath et al., 2004).

The idea of designing surrogates of E1S as STS inhibitors was revisited by Taylor et al. Several E1S and estradiol sulfate analogues, in which the sulfate group was replaced with an α,α -difluoromethylenesulfonate group (eg., **5l**, Fig. 5) or an α,α -difluoromethylenetetrazole (eg. **5m**, Fig. 5) group, were examined as inhibitors of STS. They were designed to be non-hydrolysable by the

enzyme, but to mimic the sulfate group of E1S. The difluoromethylene moiety was introduced to replace the labile oxygen in steroid sulfates and was shown to play a significant influence in inhibitory activity since compounds bearing this moiety are 4.5–10.5 times more potent than their non-fluorinated analogues as competitive inhibitors in a human placental STS preparation purified to apparent homogeneity. Compound **5m** exhibits an affinity for STS approaching that of the natural STS substrate, estrone sulfate (Lapierre et al., 2004).

Taylor et al. further applied the above design strategy to EMATE and synthesized the analogue **5n** (Fig. 5), which bears an α,α -difluorosulfonamide moiety at the 3-position on the A-ring of estrone (Liu et al., 2005). The replacement of the bridging *O*-atom of the sulfamate group in EMATE with an α,α -difluoromethylene moiety was designed to render the resulting analogue non-hydrolyzable, but yet to still mimic EMATE. Compound **5n** was found to be a reversible inhibitor with a greater affinity for STS than its non-fluorinated analogue, ie. estra-1,3,5-(10)-triene-17-one-3-methanesulfonamide. The ability of **5n** to inhibit STS was found to be pH dependent which may be attributed to a combination of the fluorines interacting with specific residues in the enzyme active site and differences in ionization state of the sulfonamide moiety and enzymatic residues.

More recently, Taylor et al. prepared several steroidal (**5o** and **5p**, Fig. 5) and non-steroidal (**5q** and **5r**, Fig. 5) boronic acids and evaluated their STS inhibitory activities using purified STS (Ahmed et al., 2006). Boronic acid **5o** (E1) was a good competitive and reversible STS inhibitor with a K_i of 2.8 μ M at pH 7.0 and 6.8 μ M at pH 8.8. The K_i of the benzyl derivative **5p** is 250 nM. Like its congener 17 α -benzylestradiol, **5p** was a potent reversible and non-competitive inhibitor. The coumarin- (**5q**) and chromenone- (**5r**) based boronic acids are modest STS inhibitors, showing IC_{50} values of 86 μ M and 171 μ M respectively. In contrast, replacing the boronic acid moiety of **5r** with a OH group yielded a good reversible, mixed-type inhibitor with a K_i of 4.6 μ M. These findings suggest that the boronic acid moiety may serve as a mimic of the sulfate group and render STS inhibitory activity if it is attached to a scaffold that closely resembles the natural substrate E1S.

It is clear that various attempts have been made to discover and design reversible inhibitors of STS. However, a clear breakthrough remains elusive and no further work has been reported recently in this area. Moreover, to the best of our knowledge that such compounds have not yet progressed to *in vivo* evaluation. Until a potent and pharmaceutically attractive reversible inhibitor is discovered, the inhibition of STS will be most effectively achieved by irreversible aryl sulfamate-based inhibitors.

Dual inhibitors

With the clear success of AIs in the clinic and the emergence of STS inhibitors as a potential effective endocrine therapy for HDBC, it has been reasoned that estrogen deprivation may be more comprehensively achieved by concurrent dual inhibition of both enzymes.

The concept of designing single agents that act against multiple biological targets is of increasing interest and prominence. In recent years, an increasing volume of work has been published exemplifying the successful use of this strategy (Morphy and Rankovic, 2005; Espinoza-Fonseca, 2006; Baraldi et al., 2007; Chen et al., 2007; Apsel et al., 2008; Meunier, 2008; Marques et al., 2008; Wei et al., 2008; Gangjee et al., 2008; Gemma et al., 2009; Gediya and Njar, 2009). We have pioneered this approach to augment our strategy directed at the first-in-class clinical target of STS inhibition and have successfully developed three series of single agent dual aromatase and sulfatase inhibitors (DASIs). The design of these DASIs shares a common principle of engendering the irreversible STS inhibitory pharmacophore (ie. an aryl sulfamate, $\text{ArOSO}_2\text{NH}_2$) into a clinical or experimental AI with minimal structural change incurred to the original scaffold in order to retain and maximize aromatase inhibition.

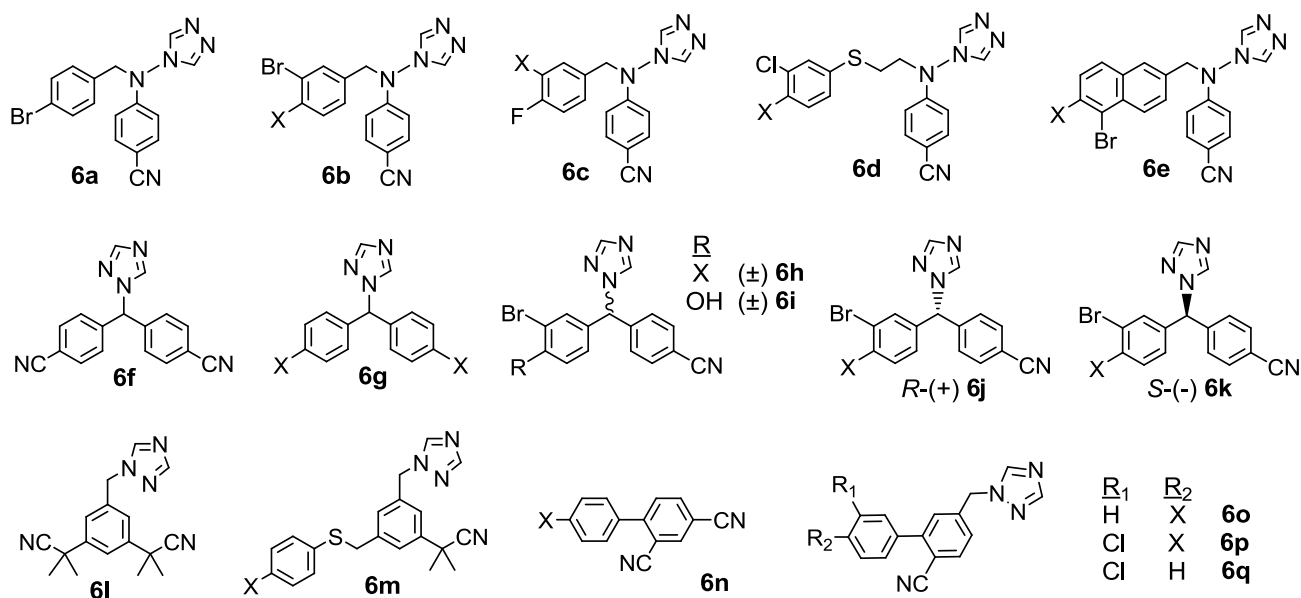


Fig. 6. DASIs derived from AIs **6a** (YM511), **6f** (letrozole) and **6l** (anastrozole); and from STS inhibitor **6n**. X = OSO₂NH₂.

The first series of DASIs was exemplified by sulfamate derivatives of the non-steroidal AI 4-((4-bromobenzyl)-[1,2,4]-triazol-4-yl-amino)benzonitrile **6a** (Fig. 6). The best DASIs *in vitro* (JEG-3 cells) are **6b** (IC₅₀_{AROM} = 0.82 nM, IC₅₀_{STS} = 39 nM), and **6c**, (IC₅₀_{AROM} = 0.77 nM, IC₅₀_{STS} = 590 nM). Both **6b** and **6c** inhibit aromatase and STS potently in PMSG pretreated adult female Wistar rats 3 h after a single oral 10 mg/kg dose. Almost complete dual inhibition is observed for **6b** but the levels are reduced to 85% (aromatase) and 72% (STS) after 24 h (Woo et al., 2003, 2007). In common with other sulfamoylated compounds, **6b** was found to complex with hCAII, suggesting its *in vivo* activity observed is likely influenced by sequestration of the inhibitor into hCAII in erythrocytes and X-ray structures of DASI-hCAII complexes have been solved (Lloyd et al., 2005b).

The methylene linker of **6b** and its congeners have recently been replaced by systems such as alkylene, thioether, ether, sulfone and sulfonamide in an SAR study. The best DASI is **6d** (Fig. 6), whose IC₅₀s against aromatase and STS are 0.45 nM and 1200 nM respectively. *In vivo*, **6d** inhibits PMSG-induced plasma estradiol levels by 92% and liver STS activity by 98% 3 h after a single oral dose of 10 mg/kg (Bubert et al., 2008).

In a very recent study, several bicyclic derivatives of **6b** and its congeners were prepared. The bicyclic ring systems investigated included tetrahydronaphthalene, naphthalene, indole, benzoxazole, and benzofuran motifs. It was found that compounds containing a sulfamoylated naphthalene ring showed the best inhibitory profile against either aromatase or STS. The most promising DASI is **6e** (Fig. 6), whose IC_{50} s against aromatase and STS are 0.25 nM and 205 nM respectively. On evaluation of oral activity *in vivo*, **6e** inhibited aromatase by 93% and STS by 93% 3 h after a single dose of 10 mg/kg (Wood et al., 2010).

A second series of DASIs are sulfamate derivatives of letrozole **6f** (Fig. 6) and bis-sulfamate **6g** was the first example synthesised which exhibited IC_{50} values of 3044 nM against aromatase and $>10\ \mu\text{M}$ against STS in JEG-3 cells (Wood et al., 2005). However, despite its apparent weak dual inhibition *in vitro*, at a single oral dose of 10 mg/kg, **6g** inhibited aromatase and rat liver STS by 60% and 88%, respectively, 24 h after administration. On recognition that retention of more structural features of letrozole in letrozole-based DASIs may improve aromatase inhibition, the racemic compound **6h** was synthesized, which inhibited aromatase and STS with IC_{50} values of 3 nM and 2600 nM in JEG-3 cells respectively. Its phenolic racemic precursor **6i** was separated by chiral HPLC, and the absolute configuration of each enantiomer was determined using vibrational and electronic circular dichroism in tandem with calculations of the predicted spectra (Wood et al., 2008; Abbate et al., 2009). On sulfamoylation of the two phenolic enantiomers, which did not initiate any racemisation, the resulting enantiopure *R*-(+)-sulfamate **6j** (Fig. 6) showed IC_{50} values of 3.2 nM (aromatase) and 4633 nM (STS), whereas the *S*-(-)-sulfamate **6k** (Fig. 6) showed IC_{50} values of 14.3 nM (aromatase) and 553 nM (STS) (Wood et al., 2008). As anticipated from previous observations (Furet et al., 1993), one enantiomer showed more potent aromatase inhibition than its antipode. For the first time, it was demonstrated that the enantiomers of a chiral sulfamate-containing compound inhibited STS to a different extent.

A third series of DASI is sulfamate derivatives of anastrozole **6l** (Fig. 6) (Jackson et al., 2007). One of the lead compounds is **6m** (Fig. 6), which inhibited aromatase ($IC_{50} = 3.5\ \text{nM}$) *in vitro* to a

similar magnitude to that of anastrozole ($IC_{50} = 1.5 \text{ nM}$), although it only showed a moderate inhibitory activity against STS ($IC_{50} > 10 \text{ }\mu\text{M}$). However, *in vivo*, **6m** surprisingly exhibited potent dual inhibition.

More recently, a different approach was adopted to design DASIs, that involves the incorporation of a reversible aromatase inhibitory pharmacophore, which is principally a heme-ligating nitrogen-containing heterocycle, into a known sulfamate-based STS inhibitor **6n** (Figure 6) (Woo et al., 2010). Several compounds were found to be good aromatase or STS inhibitors and DASI **6o** (IC_{50} : aromatase, 2.0 nM; STS, 35 nM) and its chlorinated congener **6p** (IC_{50} : aromatase, 0.5 nM; STS, 5.5 nM) were examples that showed exceptional dual potency in JEG-3 cells. Both biphenyl derivatives share a *para*-sulfamate-containing ring B and a ring A, which contains a triazol-1-ylmethyl *meta* to the biphenyl bridge and *para* to a nitrile. At 1 mg/kg po, **6o** and **6p** reduced plasma estradiol levels strongly and inhibited liver STS activity potently *in vivo*. Compound **6p** was found to be non-estrogenic in a uterine weight gain assay. It inhibits hCAII potently ($IC_{50} = 86 \text{ nM}$) and a complex of **6p** with hCAII was resolved by X-ray crystallography (Woo et al., 2010). Interestingly, in a separate development the biphenyl motif was successfully exploited for designing a new class of highly potent non-steroidal AIs (Jackson et al., 2008). A particularly potent example is **6q** (Fig. 6), the IC_{50} value of which against aromatase in JEG-3 cells was found to be 0.22 nM.

The search for dual inhibitors inhibiting STS and another target has not been confined just to DASIs. One compound, SR16157, was reported to be a novel dual-acting inhibitor of STS and the estrogen receptor. SR16157 inhibits the hydrolysis of estrone sulfate to estrone, and is cleaved by STS to SR16137 which is a tissue-selective estrogen receptor modulator, with potent antiestrogen effects in breast and uterus but protective estrogen-like agonist effects on bone and cardiovascular systems (Sambucetti et al., 2009).

Summary

A great deal of effort has been put into the discovery of STS inhibitors in the past two decades, after recognising that the hydrolysis of steroid sulfates such as E1S to their unconjugated steroids by STS may be an importance source of estrogens. Initially, attempts were focused upon preparing sulfated derivatives and sulfate surrogates of E1S, but these approaches did not yield any promising agents. The breakthrough came with the discovery of estrone 3-*O*-sulfamate (EMATE), an orally active and irreversible inhibitor of STS. Despite its highly potent in vitro and in vivo inhibitory activities against STS, the undesirable estrogenic property of EMATE prevented its further development as an anti-endocrine agent for the treatment of HDBC, although its estradiol congener entered clinical trials directed at hormone replacement therapy. Various attempts were made to design analogues of EMATE that were less estrogenic or devoid of estrogenicity, with a few successful examples being discovered. At the same time, a major effort was placed on designing non-steroidal STS inhibitors that contain an aryl sulfamate moiety, the key pharmacophore for potent irreversible inhibition of STS. Many structurally diverse compounds have been reported, although the most successful and well-studied non-steroidal STS inhibitor is STX64 which successfully underwent a Phase I trial in patients with advanced breast cancer and is currently in further such trials as well as undergoing Phase II trials for other hormone-dependent cancers.

In pursuit of an alternative mechanism for inhibiting STS, several groups pursued the design of steroidal and non-steroidal reversible inhibitors. However, the compounds reported to date did not show a level of inhibitory activity that could potentially rival that of aryl sulfamate-based irreversible inhibitors.

The future of delivering such endocrine therapy might not involve the inhibition of just one target, but of multiple ones. It has been reasoned that dual inhibition of aromatase and STS will render a more comprehensive and effective estrogen deprivation for the treatment of HDBC. While using a combination of an AI and for example the STS inhibitor STX64 as individual agents is a logical choice of treatment regime, the design of dual aromatase and sulfatase inhibitors (DASIs) as a single agent is an attractive strategy which may possess certain pharmaceutical advantages. To this

end, four different series of non-steroidal DASIs have been pursued, three of which have the aryl sulfamate pharmacophore incorporated into known AIs, while a fourth series comprises derivatives of a known STS inhibitor incorporating a heme-ligating heterocyclic ring. Potent in vitro and in vivo biological activities were observed and some of the lead compounds warranted further investigation for their potential as a therapeutic agent for the treatment of hormone-dependent cancers.

Significant progress has been made in the past two decades in discovering and synthesizing STS inhibitors for clinical development. The aryl sulfamate pharmacophore has been at the heart of these developments. With proof-of-concept trials now in progress, it may take another decade before the full therapeutic potential of STS inhibition can be fully evaluated and appreciated.

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